

## University of Groningen

### **In vivo labeling of rabbit cholinergic basal forebrain neurons with fluorochromated antibodies**

Hartig, W; Varga, C; Kacza, J; Grosche, J; Seeger, J; Luiten, PGM; Brauer, K; Harkany, T;  
Härtig, Wolfgang

*Published in:*  
Neuroreport

**IMPORTANT NOTE:** You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

*Document Version*  
Publisher's PDF, also known as Version of record

*Publication date:*  
2002

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

Hartig, W., Varga, C., Kacza, J., Grosche, J., Seeger, J., Luiten, PGM., Brauer, K., Harkany, T., & Härtig, W. (2002). In vivo labeling of rabbit cholinergic basal forebrain neurons with fluorochromated antibodies. *Neuroreport*, 13(11), 1395-1398.

#### **Copyright**

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

#### **Take-down policy**

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

*Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.*

# *In vivo* labeling of rabbit cholinergic basal forebrain neurons with fluorochromated antibodies

Wolfgang Härtig,<sup>CA</sup> Csaba Varga, Johannes Kacza,<sup>1</sup> Jens Grosche, Johannes Seeger,<sup>1</sup> Paul G. M. Luiten,<sup>2</sup> Kurt Brauer and Tibor Harkany<sup>2,3</sup>

Paul Flechsig Institute for Brain Research, University of Leipzig, Jahnallee 59, D-04109 Leipzig; <sup>1</sup>Department of Anatomy, Histology and Embryology, University of Leipzig, An den Tierkliniken 43, D-04103 Leipzig, Germany; <sup>2</sup>Department of Molecular Neurobiology, University of Groningen, Kerklaan 30, NL-9750 AA Haren, The Netherlands; <sup>3</sup>Department of Medical Chemistry, Faculty of Medicine, University of Szeged, Szeged, Hungary

<sup>CA</sup>Corresponding Author

Received 2 May 2002; accepted 9 May 2002

Cholinergic basal forebrain neurons (CBFN) expressing the low-affinity neurotrophin receptor p75 (p75<sup>NTR</sup>) were previously selectively labeled *in vivo* with carbocyanine 3 (Cy3)-tagged anti-p75<sup>NTR</sup>, but the applied 192IgG-conjugates recognized p75<sup>NTR</sup> only in rat. The antibody ME 20.4 raised against human p75<sup>NTR</sup> had been shown to cross-react with the receptor in monkey, raccoon, sheep, cat, dog, pig and rabbit. Hence, for *in vivo* labeling of rabbit CBFN in the present study, ME 20.4 was fluorochromated with Cy3-N-hydroxysuccinimide ester and purified Cy3-ME 20.4 was injected

intracerebroventricularly. Two days post-injection, clusters of Cy3-ME 20.4 were found in CBFN displaying choline acetyltransferase-immunoreactivity. Following photoconversion, electron microscopy revealed fluorochromated antibodies in secondary lysosomes. In conclusion, Cy3-ME 20.4 might become an appropriate marker for CBFN in live and fixed tissues of various mammalian species. *NeuroReport* 13:1395–1398 © 2002 Lippincott Williams & Wilkins.

**Key words:** Choline acetyltransferase; Cholinergic system; Low-affinity neurotrophin receptor; Oxygen-enriched photoconversion

## INTRODUCTION

Severe damage to cholinergic neurons of the basal forebrain (BFB) is a major neuropathological hallmark of Alzheimer's disease, and this has prompted investigations focused on this brain region in various mammalian species [1]. Neuroanatomical studies have achieved the identification of cholinergic nerve cells by the detection of their marker enzyme choline acetyltransferase (ChAT, EC 3.2.1.6) as well as of vesicular acetylcholine transporters. An additional selective marker of cholinergic BFB neurons is the low-affinity neurotrophin receptor p75 (p75<sup>NTR</sup>), as was demonstrated in the rat [2], raccoon [3], monkey [4] and human [5]. Targeting of p75<sup>NTR</sup> led to the development of cholinergic immunolesion models. In particular, the *in vivo* application of antibodies directed against an extracellular epitope of rat p75<sup>NTR</sup> and conjugated to the ribosome-inactivating toxin saporin (192IgG-saporin) caused selective lesions of cholinergic projection neurons in the rat BFB [6].

A further derivative of 192IgG, namely its conjugate with the red fluorochrome carbocyanine 3 (Cy3-192IgG), was introduced as a tool for the selective labeling of rat cholinergic, p75<sup>NTR</sup>-bearing neurons both *in vivo* [7,8] and *in vitro* [9]. 192IgG-conjugates are commonly infused intracerebroventricularly and bind to p75<sup>NTR</sup> on cholinergic terminals, become internalized as ligand/receptor complexes and are transported retrogradely to the perikarya of cholinergic BFB neurons [6]. Internalized Cy3-192IgG

molecules form clusters in secondary lysosomes [8]. Whereas ribosome inactivation by saporin causes neuronal death [6,10], Wu *et al.* [11] demonstrated that pre-labeling of cholinergic neurons with Cy3-192IgG does not alter their electrophysiological and pharmacological properties in *ex vivo* brain slice preparations containing the medial septum/diagonal band complex (MSDB). Concordantly, identification of cholinergic nerve cells with Cy3-192IgG in brain slices led to new insights in the synaptic organization of neurons in the MSDB with implications for learning and memory [11,12].

However, 192IgG conjugates recognize p75<sup>NTR</sup> only in the rat precluding their application in other mammalian species (e.g. monkeys) with a BFB chemoarchitecture resembling that in humans [13]. In contrast, the monoclonal antibody ME 20.4 raised against an extracellular epitope of the human p75<sup>NTR</sup> [14] was demonstrated to cross-react with p75<sup>NTR</sup> also in monkey, raccoon [3], rabbit, pig, dog and cat [15]. Accordingly, the ME 20.4-saporin conjugate was shown to be a selective cholinotoxin in the common marmoset [16], rhesus monkey [17], rabbit [18] and sheep [19].

To overcome the restriction of *in vivo* labeling in the rat, the present study investigated whether Cy3-ME 20.4 can be employed as a marker of living cholinergic neurons in a wide range of mammalian species expressing analogues of the human p75<sup>NTR</sup>. Encouraged by recent data on ME 20.4-saporin-induced immunolesions of cholinergic cells and

subsequent  $\beta$ -amyloid deposition in the rabbit brain [18], the potential of Cy3-ME 20.4 as a label for cholinergic nerve cells was exemplified in studies on BFB neurons of this animal species.

## MATERIALS AND METHODS

**Preparation of Cy3-ME 20.4:** Cy3-ME 20.4 was prepared by coupling the antibody with a chemically activated ester of Cy3 under reaction conditions proved to maintain the antigen-binding properties of ME 20.4 [20]. Four hundred micrograms of ME 20.4 (pure IgG; AB-N07, Advanced Targeting Systems, San Diego, CA, USA) were reacted with a monofunctional Cy3-N-hydroxysuccinimide ester (Antibody Cy3 Labeling Kit, Amersham Pharmacia Biotech, Freiburg, Germany) according to the instruction of the manufacturer at pH 9.3 for 1 h, but with doubled dye:protein ratio. Thereafter, the reaction mixture was dialyzed against phosphate-buffered saline ( $3 \times 4$  h) and the concentration of Cy3-ME 20.4 was adjusted to approximately 0.5 mg/ml prior its use for intracerebroventricular (i.c.v.) injections.

**Surgical procedure:** New Zealand white rabbits of either sex ( $n = 5$ ; 2.3–5.1 kg; 4–21 months of age) were used in this study. The rabbits were anesthetized with a mixture of ketamine (50 mg/kg; Exalgon, Merck, Hallbergmoos, Germany) and xylazine (4 mg/kg; Rompun, Bayer AG, Leverkusen, Germany), and their heads were mounted in a stereotaxic frame. Two animals received unilateral i.c.v. injections, and three animals were injected bilaterally with 16  $\mu$ l Cy3-ME 20.4 at an infusion speed of 0.5  $\mu$ l/min with a Hamilton microsyringe (Hamilton, Bonaduz, Switzerland). Injection co-ordinates were applied according to Beach *et al.* [18] at AP = 0.0 mm, L = 2.2 mm and DV = 9.0/7.5 mm relative to bregma. All efforts were made to minimize animal suffering throughout the experiments including administration of the analgetic Metamizol (150 mg/kg; Berlosin, Berlin-Chemie, Germany) immediately after operation. The antibiotic Enrofloxacin (10 mg/kg daily; Baytril, Bayer AG) was given orally throughout the post-injection period. The care and treatment of the animals were in accordance with the European Communities Council Directive (86/609/EEC) and approved by the Laboratory Animal Care and Use Committee of the University of Leipzig (TVV-Nr.15/00).

**Immunocytochemistry and electron microscopy:** Two days post-injection rabbits were transcardially perfused with 4% paraformaldehyde containing either 0.5% glutardialdehyde (three animals) or 0.1% glutardialdehyde (two animals) in 0.1 M phosphate-buffer (pH 7.4). Whole brains were removed from the skulls, divided into fore- and hindbrain regions and post-fixed overnight in 4% paraformaldehyde. Two forebrains were coronally sectioned on a vibratome at 50  $\mu$ m and the remaining tissue was cryoprotected and cut at 30  $\mu$ m on a cryostat microtome. Sections spanning the MSDB and the anterior subdivision of the nucleus basalis magnocellularis were washed with Tris-buffered saline (TBS, 0.1 M, pH 7.4), rinsed in distilled water, mounted, air-dried and coverslipped with Entellan (in toluene; Merck, Darmstadt, Germany).

To determine the specificity of *in vivo* labeling, Cy3-ME 20.4 pre-labeled sections were applied to the concomitant immunolabeling of ChAT and the calcium-binding protein calretinin. Non-specific binding sites for the immunoreagents were primarily blocked with 5% normal donkey serum in 0.1 M Tris-buffered saline (TBS) containing 0.3% Triton X-100 for 1 h. Subsequently, the sections were incubated with a mixture of affinity-purified goat anti-ChAT (1:25; AB144P, Chemicon International; Hofheim, Germany) and rabbit anti-calretinin (1:300; AB149, Chemicon) diluted in the blocking solution for 16 h. Next, rinsed sections were reacted with a cocktail of Cy2-tagged donkey anti-goat IgG and Cy5-conjugated donkey anti-rabbit IgG (20  $\mu$ g/ml; Dianova, Hamburg, Germany) diluted in TBS containing 2% bovine serum albumin for 1 h. Omission of the primary antibodies in control experiments led to the absence of any cellular Cy2- and Cy5-labeling. After the cytochemical procedures the sections were rinsed, mounted, air-dried and coverslipped in Entellan. Fluorescence microscopy was performed using an Axioplan microscope (Zeiss, Oberkochen, Germany) and selected sections were inspected with a Zeiss 510 confocal laser-scanning microscope.

To quench the autofluorescence caused by lipophilic lipofuscin-like compounds in sections from 21-month-old rabbits, tissue was treated with Sudan Black B according to Schnell *et al.* [21] but prior to the immunofluorescence labeling of cholinergic neurons. All Sudan Black B-pre-treated sections were embedded in glycerol/gelatin (Sigma, Deisenhofen, Germany).

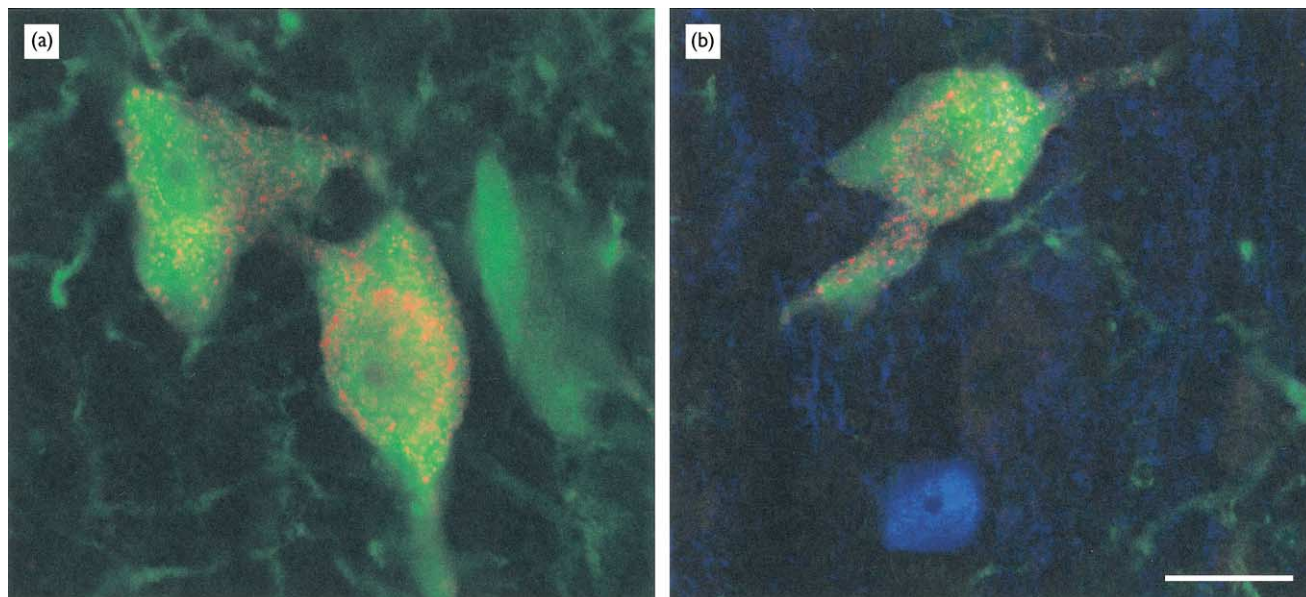
The subcellular distribution of Cy3-ME 20.4 was determined following photoconversion of Cy3-ME 20.4 into an electron-dense reaction product applying oxygen-enriched photoconversion in a closed conversion chamber as described previously [8]. Specimens were analyzed with a Zeiss 900 electron microscope.

## RESULTS

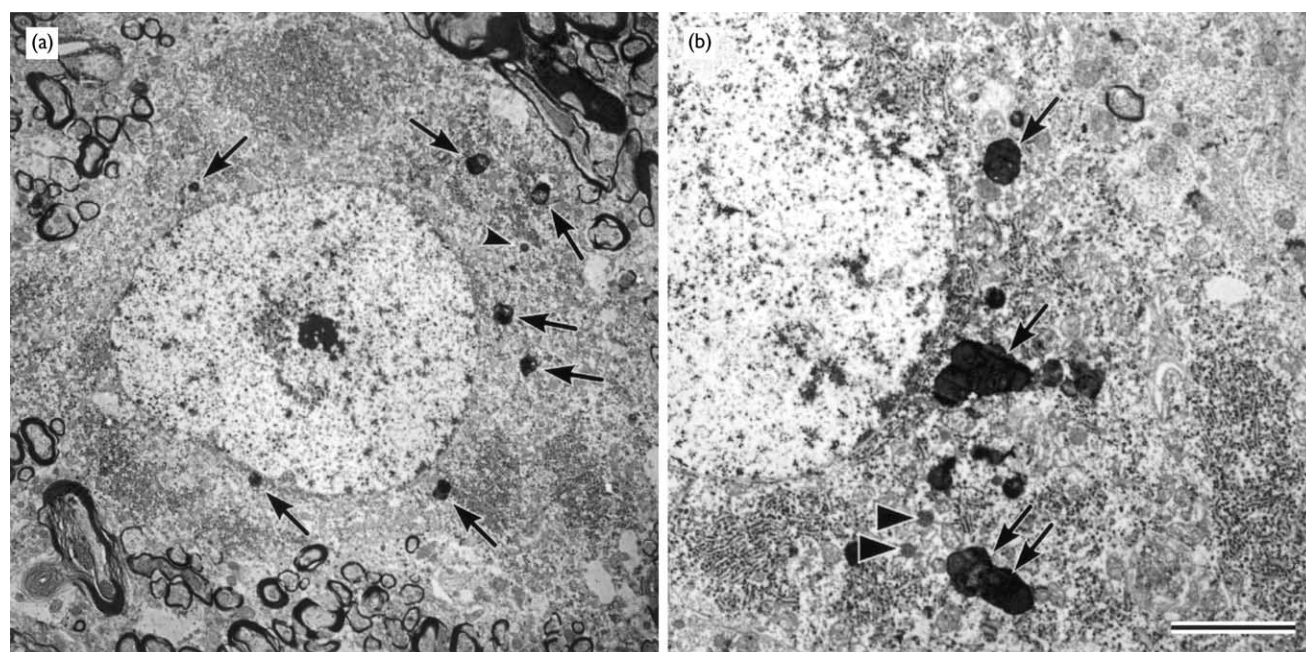
Two days following the i.c.v. infusion of Cy3-ME 20.4 there was strong punctate labeling in many cells of the MSDB (Fig. 1) that became less intense in the nucleus basalis magnocellularis. This staining pattern was observed irrespective of the concentration of glutardialdehyde in the fixative. In comparison to rabbits unilaterally infused with Cy2-ME 20.4, bilaterally injected animals appeared to contain more *in vivo*-labeled nerve cells in both hemispheres.

*In vivo* labeling of Cy3-ME 20.4 was exclusively found in cholinergic neurons, as revealed by their immunoreactivity for ChAT (Fig. 1a) and p75<sup>NTR</sup> (not shown). In contrast, *in vivo* labeling was never observed in non-cholinergic, calretinin-immunoreactive nerve cells (Fig. 1b) that are known to be intermingled with cholinergic neurons in the border zone of the medial and lateral septum [22] and in magnocellular, parvalbumin-containing GABAergic BFB neurons (not shown).

It is noteworthy that treatment of the sections from 21-month-old rabbits with Sudan Black B quenched the autofluorescence caused by lipophilic lipofuscin-like compounds. Importantly, Sudan Black B treatment only slightly diminished the intensity of *in vivo* labeling with hydrophilic



**Fig. 1.** The confocal laser-scanning micrograph (a) shows red punctate Cy3-ME 20.4 *in vivo* labeling in cholinergic neurons of the rabbit medial septum. Selectivity of Cy3-ME 20.4 for cholinergic neurons was demonstrated by co-localization with choline acetyltransferase (ChAT) that appears green (Cy2). (b) Merged carbocyanine triple fluorescence staining of Cy3-ME 20.4 (red clusters) and immunoreactivities for ChAT (Cy2, green) and calretinin (Cy5, color-coded in blue) in the border zone between the medial and lateral septum confirmed the specificity of *in vivo* labeling for cholinergic neurons and its absence in non-cholinergic, calretinin-containing nerve cells. Bar = 25  $\mu$ m.



**Fig. 2.** Electron micrographs of cholinergic neurons in the medial septum (a) and, at higher magnification, in the diagonal band of Broca (b) following photoconversion of internalized Cy3-ME 20.4. The *in vivo* label was predominantly present in secondary lysosomes (arrows) after two days of survival. In comparison to the photoconverted material, non-labeled primary lysosomes (arrowheads) are different in their size, shape and electron density. Prior to print, the brightness and contrast of the electron micrographic images were digitally adjusted and shading correction was performed to reduce the effects of uneven illumination. Bar = 500 nm (a), 250 nm (b).

Cy3 and of the subsequently performed Cy2-immunostaining of cholinergic neurons.

Electron microscopic analysis elucidated that Cy3-ME 20.4 was predominantly located intracellularly in secondary lysosomes (Fig. 2a,b).

## DISCUSSION

The granular cellular labeling of neurons in the rabbit BFB with i.c.v. applied Cy3-ME 20.4 resembled that of Cy3-192IgG in the rat BFB [7]. A subpopulation of cholinergic neurons in the MSDB was unlabeled by Cy3-ME 20.4 that also parallels the incomplete Cy3-192IgG labeling in the rat [7,11]. Recently, the counting of Cy3-192IgG-positive cells in the rat BFB revealed a medial septum  $\geq$  horizontal limb of the diagonal band  $>>$  nucleus basalis magnocellularis gradient of labeling efficacy [23]. An apparently similar gradient was observed after the *in vivo* labeling with Cy3-ME 20.4; that, however, remains to be quantified. Future studies should also elucidate whether the *in vivo* labeling with Cy3-ME 20.4 is long-term stable or undergoes gradual clearance from the neurons, as was previously shown for Cy3-192IgG in the rat [8].

The predominant location of Cy3-ME 20.4 in secondary lysosomes, as revealed by electron microscopy, is identical to the ultrastructural localization of Cy3-192IgG in rat basal forebrain neurons [8]. This finding also corresponds with the observation that lysosomes are preferred targets for internalized nerve growth factor [24], the natural ligand of p75<sup>NTR</sup>, which might be mimicked by fluorochromated antibodies raised against p75<sup>NTR</sup>.

Whereas the quenching of autofluorescence with Sudan Black B in the present work hardly affected the intensity of *in vivo* labeling and subsequent immunofluorescence staining, strong autofluorescence might interfere with the identification of cholinergic neurons with Cy3-ME 20.4 in live brain slices originating from aged animals.

Our present findings suggest the potential use of Cy3-ME 20.4 in future neuropharmacological studies targeting the cholinergic system of higher mammals. Such investigations are also emphasized by the accelerated gathering of pharmacological data based on the *in vivo* labeling of rat cholinergic basal forebrain neurons [11,12]. In particular, these studies showed that muscarinic receptor antagonists induce memory impairment due to a decrease of septo-hippocampal GABA release, instead of the previously assumed diminished hippocampal acetylcholine release, and confirmed neuroanatomical data on collaterals of cholinergic neurons contacting parvalbumin-immunopositive GABAergic neurons within the MSDB [25].

## CONCLUSION

The high interspecies variability of the chemical nature, structure and topography of the cholinergic components in magnocellular BFB nuclei hampers the development of general functional concepts based on investigations of only one or a few animal species. Therefore, the use of ME 20.4-saporin conjugates both in primates [16,17] and in sheep [19] suggests that Cy3-ME 20.4 might be successfully employed also in primates and ungulates, and become a useful cholinergic marker to determine morphological, electrophysiological and pharmacological parameters of cholinergic neurons under physiological or pathological conditions.

## REFERENCES

1. Záborszky L, Pang K, Somogyi J *et al.* *Ann NY Acad Sci* **77**, 339–367 (1999).
2. Springer JE, Koh S, Tayrien MW and Loy R. *J Neurosci Res* **17**, 111–118 (1987).
3. Tremere L, Brückner G, Brauer K *et al.* *Brain Res* **797**, 351–356 (1998).
4. Kordower JH, Bartus RT, Bothwell M *et al.* *J Comp Neurol* **277**, 465–486 (1988).
5. Hefti F, Hartikka J, Salvaterra P *et al.* *Neurosci Lett* **69**, 275–281 (1986).
6. Wiley RG. *Trends Neurosci* **15**, 285–290 (1992).
7. Härtig W, Seeger J, Naumann T *et al.* *Brain Res* **808**, 155–165 (1998).
8. Kacza J, Grosche J, Seeger J *et al.* *Brain Res* **867**, 232–238 (2000).
9. Ha DH, Robertson RT and Weiss JH. *J Neurosci* **18**, 4201–4215 (1998).
10. Rossner S. *Int J Dev Neurosci* **15**, 835–850 (1997).
11. Wu M, Shanabrough M, Leranthe C and Alreja M. *J Neurosci* **20**, 3900–3908 (2000).
12. Alreja M, Wu M, Liu W *et al.* *J Neurosci* **20**, 8103–8110 (2000).
13. Geula C, Schatz C-R and Mesulam M-M. *Neuroscience* **54**, 461–476 (1993).
14. Ross AH, Grob P, Bothwell M *et al.* *Proc Natl Acad Sci USA* **81**, 6681–6685 (1984).
15. Tremere LA, Pinaud R, Grosche J *et al.* *Neuroreport* **11**, 2177–2183 (2000).
16. Fine A, Hoyle C, Maclean CJ *et al.* *Neuroscience* **81**, 331–343 (1997).
17. Mrzljak L, Levey A, Belcher S and Goldman-Rakic PS. *J Comp Neurol* **390**, 112–132 (1998).
18. Beach TG, Potter PE, Kuo Y-M *et al.* *Neurosci Lett* **283**, 9–12 (2000).
19. Ferreira G, Meurisse M, Tillet Y and Lévy F. *Neuroscience* **104**, 419–439 (2001).
20. Härtig W and Fritschy J-M. Immunofluorescence: conjugation of dyes to antibodies. In: *Embryonic Encyclopedia of Life Sciences*. London: Nature Publishing Group; 2000. www.els.net, pp. 1–3.
21. Schnell SA, Staines WA and Wessendorf MW. *J Histochem Cytochem* **47**, 719–730 (1999).
22. Jakab RL and Leranthe C. Septum. In: Paxinos G, ed. *The Rat Nervous System*. London: Academic Press; 1995, pp. 405–442.
23. Harkany T, Grosche J, Mulder J *et al.* *Neuroscience* **108**, 611–627 (2001).
24. Kasaian MT and Neet KE. *J Biol Chem* **263**, 5083–5090 (1988).
25. Brauer K, Seeger G, Härtig W *et al.* *J Neurosci Res* **54**, 248–253 (1998).

**Acknowledgements:** The excellent technical assistance of Mrs Ute Bauer, Mrs Gabriele Lindner and Mrs Gudrun Lemm is gratefully acknowledged. This study was supported by the Deutsche Forschungsgemeinschaft (grant Ha 2211/2-1 to W.H. and C.V.), the Interdisziplinäres Zentrum für Klinische Forschung (IZKF) at Leipzig University (grant 01 KS 9504 projects Z10 and C5 to J.G. and J.S., respectively) and the Hungarian National Science Foundation (OTKA, F035254, to T.H.).